GC-MS Characterisation of Sapogenins from Sisal Waste and a Method to Isolate Pure Hecogenin

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Five steroidal sapogenins (tigogenin, neotigogenina, hecogenin, gloriogenin, and dehydrohecogenin) were characterised by gas chromatography coupled with mass spectrometry (GC-MS) from a hydrolysed extract of sisal waste. In addition, pure hecogenin, an important raw material for the pharmaceutical industry, was obtained from this waste by selective liquid-liquid extraction of saponins with only hecogenin as aglycone, followed by acid hydrolysis. The yield of pure hecogenin was 460 mg.Kg^{-1} of sisal waste.

Keywords: Agave sisalana; Sisal waste; Extraction; Steroids; Hecogenin

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INTRODUCTION

Steroidal sapogenins are a glycone non-sugar portion of the saponin molecule used for the semi-synthesis of bioactive compounds. Example compounds used in this application include the following: smilagenin, sarsasapogenin, diosgenin, yamogenin, tigogenin, neotigogenin, gloriogenin, gentrogenin, hecogenin, sisalagenin, 9-dehydro-hecogenin, and gitogenin (Agrawal *et al.* 1985). Among these steroidal sapogenins, diosgenin, sarsasapogenin, and hecogenin are particularly important. The usefulness of hecogenin (Fig. 1) as a synthetic starting material is due to the presence of an oxygen atom in the C-12 position that can be moved to the C-11 position. This makes it possible to introduce the 9-11 double bond required for the syntheses of corticosteroids (Beauvoir 1976).

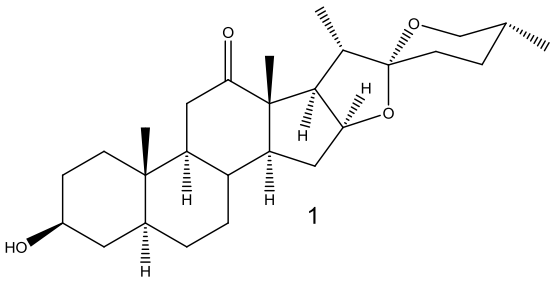

Fig. 1. Chemical structural of hecogenin

In the 1940s, steroidal sapogenins achieved great economic importance because of their transformation into pharmaceutically valuable derivatives such as corticosteroids (prednisone, dexamethasone, betamethasone, triamcinolone, and others), sexual hormones, and steroid diuretics (Fernández-Herrera *et al.* 2009).

Several researchers have demonstrated that hecogenin is useful in other applications, for example, antiproliferative activity and induction of apoptosis in several cell lines (Corbière *et al.* 2003); synthesis of glucosamine derivatives of the steroidal sapogenins (diosgenin and hecogenin) with antiproliferative and selective activity against HeLa, CaSki, and ViBo cervico-uterine cancer cells (Fernández-Herrera *et al.* 2012); obtaining dinorcholanic lactones, which are potentially useful starting materials for the synthesis of bioactive steroids from steroidal sapogenins (Ruíz-Pérez *et al.* 2012); and obtaining new brassinosteroid analogs (Gómez-Calvario *et al.* 2013)

Sisal (*Agave sisalana* Perrine) is known worldwide as a source of hard fibres, and Brazil is responsible for more than 68% of the world's production. However, only 3 to 5% of the leaves are utilised in the production of fibres; the remainder (95 to 97%) of the processing waste is used as organic fertiliser and animal feed (Santos 2006).

There has been considerable interest in this plant following the discovery in 1951 that *A. sisalana* leaves contain hecogenin. After hecogenin, the most abundant steroidal compound in mature leaves is tigogenin. The hecogenin-to-tigogenin ratio is commercially important because the proportion of tigogenin should be as low as possible (Cripps and Blunden 1978), and the tigogenin do not have the carbonyl function at C-12 position, discouraging its use due to an increased number of steps in the synthesis of pharmaceuticals derivatives. However, there are few studies on the sapogenins of sisal waste. In this work, sapogenins were analysed by GC-MS and pure hecogenin was isolated from sisal waste.

EXPERIMENTAL

General Experimental Procedures

Gas chromatography (GC) analyses were performed on a Shimadzu GC-2010 series apparatus fitted with an FID detector employing the following conditions: capillary column Rtx® -1 (30 m × 0.25 mm × 0.25 mm, composed of 100% dimethylpoly-siloxane); injector temperature of 310 °C; N_2 was used as the carrier gas at a constant flow of 0.71 mL; an injection volume of 1 μL (split ratio: 20/1); and a detector temperature of 320 °C. The oven temperature was programmed with 320 °C isotherms for 20 minutes.

GC coupled with mass spectrometry (GC-MS) analyses were performed on a Shimadzu GCMS-2010 series (electron impact ionisation at 70 eV). Helium was used as the carrier gas at a constant flow of 0.92 mL, and the general analysis conditions were as follows: capillary column Rtx® -5 (30 m × 0.25 mm × 0.25 mm, composed of 5% diphenyl 95% dimethylpolysiloxane); injector temperature of 240 °C; and an injection volume of 1 μL was employed (split ratio: 20/1). The oven temperature was programmed to start at 150 °C with an increase of 30 °C/min to 270 °C (for 10 min), then to increase at a rate of 30 °C/min to 300 °C, ending with a 14-min isotherm at 300 °C (30 min).

The ^{13}C NMR spectra were obtained on a Bruker AC-200 (100 MHz) device using deuterated chloroform ($CDCl_3$) and tetramethylsilane as the solvent and internal reference, respectively. The chemical shift values were measured in ppm.

Acquisition of Sisal Waste

The sisal waste was collected directly from a decortication machine on a sisal farm in the city of Valente, Bahia state, Brazil (S 11° 24' 53"). The processed sisal leaves were from plants six years of age that were collected in May 2012. One kilogram of sisal waste was refluxed in distilled water (1 L) for 3 h. After this procedure, the extract was filtered and concentrated in a forced circulation oven at 50 °C to yield 8.7% crude sisal waste extract.

Obtaining standard hecogenin

The crude extract was refluxed in ethanol (95%) at 100 °C for 12 h to yield ethanol extract, which was hydrolysed with HCl according to the methodology described by Harborne (1998). The hydrolysed mixture was neutralized with NaOH and extracted with hexane and purified after successive open column chromatographs and recrystallisations with acetone to yield hecogenin. ^{13}C NMR data of 3-β-hydroxy-(25*R*)-5α-spirostan-12-one (100.5 MHz, $CDCl_3$) are showed in Table 1. These data are in accordance with the literature (Agrawal *et al.* 1985).

Table 1. ^{13}C NMR (100.5 MHz, $CDCl_3$) Data of Hecogenin

Position	hecogenin δ_C	Reference δ_C	Position	hecogenin δ_C	Reference δ_C
1	36.56	36.5	16	79.27	79.1
2	31.48	31.2	17	53.59	53.5
3	70.99	70.7	18	16.09	16.0
4	37.95	37.8	19	12.04	12.0
5	44.71	44.6	20	42.26	42.2
6	28.36	28.2	21	13.33	13.3
7	31.63	31.4	22	109.33	109.0
8	34.43	34.4	23	31.29	31.2
9	55.59	55.5	24	28.85	28.8
10	36.16	36.0	25	30.26	30.2
11	37.95	37.8	26	66.96	66.8
12	213.66	213.0	27	17.19	17.1
13	55.18	55.0			
14	55.87	55.8			
15	31.63	31.5			

Obtaining pure hecogenin

The crude extract was re-suspended in an ethanol/water solution (80:20) and maintained at room temperature for 18 h to remove the polysaccharides (Izydorczyk 2005; Santos *et al.* 2013). After this time, the precipitated polysaccharides were separated by filtration and the hydroalcoholic extract (without polysaccharides) was concentrated (Fig. 2).

The hydroalcoholic extract was partitioned with ethyl acetate (1:2, v/v, 2 times) and the organic phase was concentrated under reduced pressure in a rotary evaporator. The ethyl acetate (1.0 g) and hydroalcoholic (1.0 g) extracts were subjected to a

hydrolysis reaction under reflux for 3 h in a hydroethanol solution 80% (30 mL) containing 5 mL of concentrated HCl. The reaction product was neutralized with NaOH and then extracted with ethyl acetate to yield the sapogenins present in the fractions, after removal of the solvent using a rotary evaporator. The hydrolysis products were reserved and dissolved in ethyl acetate at a concentration of 0.5 mg/mL.

RESULTS AND DISCUSSION

Steroidal sapogenins usually exist as their glycosides, also known as C-27 steroidal saponins. These compounds can be classified into two major subclasses: spirostanol and furostanol saponins. According to the literature, these compounds are synthesised by, species of the families such as Liliaceae, Convallariaceae, Dioscoreaceae, Agavaceae, Smilacaceae, and Amaryllidaceae (Yang *et al.* 2006).

The scheme for obtaining total sapogenins and pure hecogenin from sisal waste is shown in Fig. 2. The analysis conditions for sisal waste sapogenins by GC were investigated using the hecogenin standard. Among the methods studied, an isotherm of 320 °C proved to be a good method, with an indicated retention time of 8.1 minutes.

GC-MS analysis of the hydrolysed hydroalcoholic extract showed total sapogenins (Fig. 3) and revealed two steroid regions: between 6 and 7 min (peaks a and b with the same molecular ion at *m/z* 416 $[M]^+$) and between 8 and 10 min (peaks d and e) at *m/z* 430 $[M]^+$. The mass difference (14 Daltons) observed between these regions was attributed to steroid non-carbonyls and carbonyls. The characteristic fragment at *m/z* 139 for spirostanol sapogenins was observed in all peaks (Djerassi 1970). The peak c with molecular ion at *m/z* 428 $[M]^+$ was observed between these two regions.

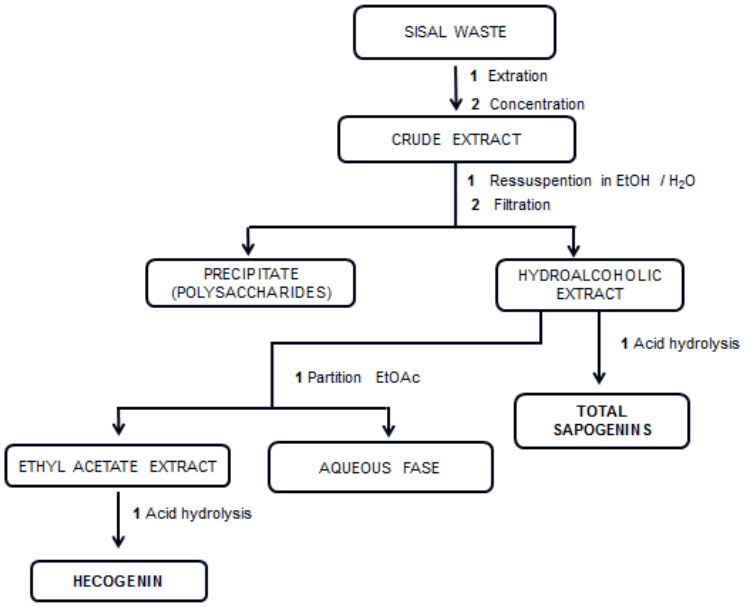

Fig. 2. Scheme for obtaining sapogenins and pure hecogenin from sisal waste

The mass spectra of peaks c, d, and e exhibited other important fragments, such as at *m/z* 126, which is intense in the 12-keto sterol. Djerassi (1970) describes this fragment

as $C_8H_{14}O$, which essentially requires that it be derived from ring F. Furthermore, the fragment was not observed in sapogenins that did not have a 12-keto functional group. A possible explanation for the increased stability is the presence of a 12-keto group in the accompanying neutral fragment, which may be formulated as the diene-dione.

Table 2 shows other important fragments that were observed during the GC-MS analyses. The peaks a and b are tigogenin {(25*R*)-5*α*-spirostan-3*β*-ol} and *neo*tigogenin {(25*S*)-5*α*-spirostan-3*β*-ol}; whereas d and e are hecogenin and gloriogenin (3-β-hydroxy-(25*R*)-5β-spirostan-12-one). The chemical structures of steroidal sapogenins obtained from sisal waste are showed in Fig. 4.

Peak c is Δ^9-hecogenin, a dehydrohecogenin. This peak showed a molecular ion at *m/z* 428 $[M]^+$ and fragments at *m/z* 126 and 139. The normal fragments (*m/z* 300, 282, 271, and 253) for a Δ^5-spirostene without nuclear substitution or for a 5α- or 5β-spirostane (*m/z* 302, 273, and 255) (Abdel-Aziz *et al.* 1990) were not observed, suggesting the presence of dehydrohecogenin, which has already been described in the literature (Higgins 1976). Peak d was characterised as hecogenin by comparison with the pure standard. Peak e was characterised as gloriogenin (Blunden *et al.* 1974a, b).

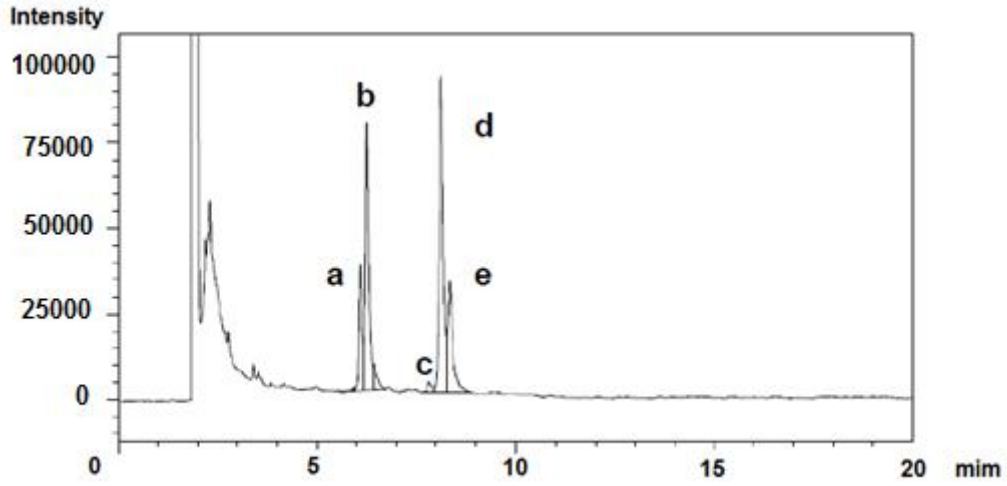

Fig. 3. GC chromatogram of hydrolysed hydroalcoholic extract from sisal waste

The ethyl acetate partition of the hydroalcoholic extract showed itself to be selective for saponins containing hecogenin as aglycone. The chromatogram of hydrolysed ethyl acetate extract from sisal waste (Fig. 5) exhibited one peak at 8.1 min. Furthermore, the ethyl acetate and hydroalcoholic extracts yielded 460.2 and 450.4 mg sapogenins per kilo of sisal waste, respectively.

Sapogenins may be obtained through the chemical, enzymatic, or hydrothermal hydrolysis of crude saponins and/or saponin-rich extracts, followed by extraction with non-polar solvents or supercritical fluids (Güçlü-Üstündağ and Mazza 2007). In the traditional method, mineral acids (*e.g.*, HCl and H_2SO_4) are usually used for this purpose (Blunden *et al.* 1974a, b).

The hecogenin-tigogenin mixture separation comprehends, initially, the acetylation of hydroxyl group in C-3 (Löken 1976). In this work, the liquid-liquid extraction by ethyl acetate showed high selectivity for hecogenin. The low solubility in

the aqueous solution of saponins that had hecogenin as an aglycone might explain the relevant selectivity of the ethyl acetate extract to obtain hecogenin.

Table 2. Ionic Fragments Observed in the Mass Spectra of Peaks Eluted in GC-MS Chromatogram of Hydrolysed Hydroalcoholic Extract from Sisal

Peak	Fragments (*m/z*) *	Compounds	References
a	416 (2) 302(15) 287(14) 273(35) 139(100) 115(18)	tigogenin	Bernardo *et al.* 1996
b	416 (2) 402(3) 302(15) 287(15) 273(38) 139(100) 115(18)	*neo*tigogenin	Bernardo *et al.* 1996
c	428 (3) 126(36) 39(100)69(25)	Δ^9-dehydrohecogenin	Blunden et al. 1974a, b; Higgins 1976
d	430 (4) 316(9) 273(14)126(30) 139(100) 69(26)	hecogenin	Djerassi 1970
e	430 (4) 402(2) 316(9) 273(14)126(30) 139(100) 69(26)	gloriogenin	Blunden *et al.* 1974a, b

*intensity %

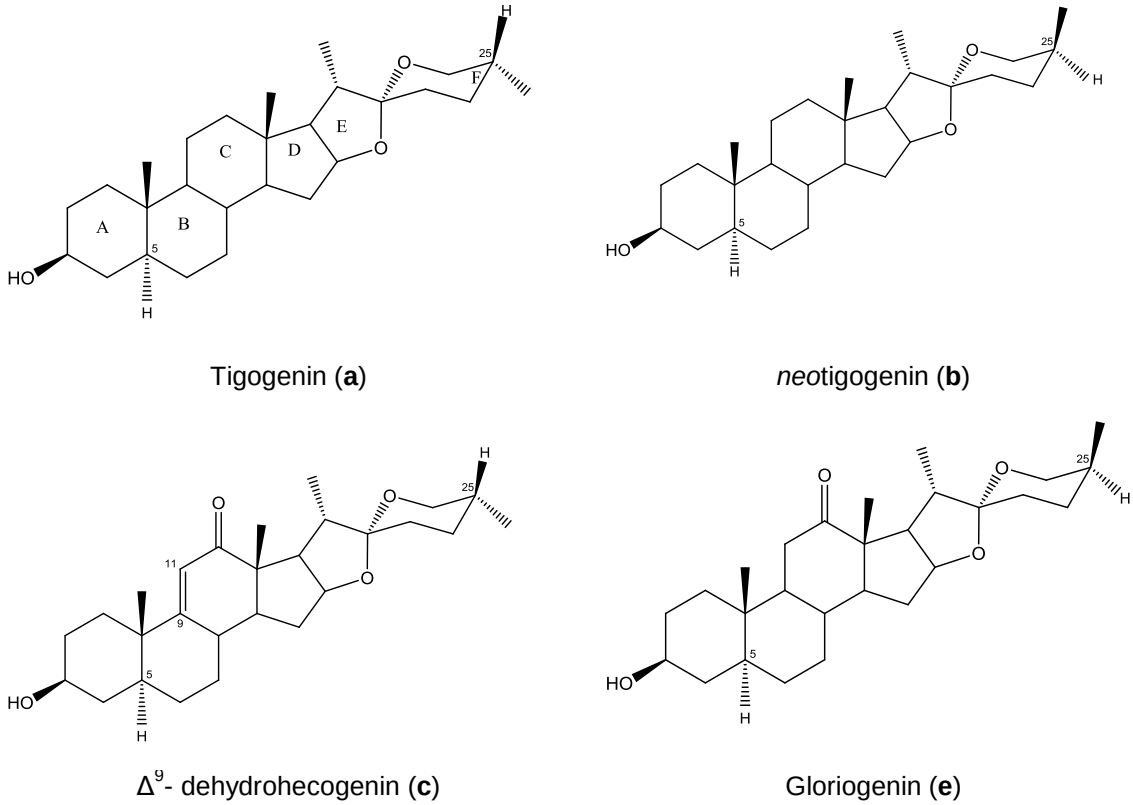

Fig. 4. Chemical structural of steroidal sapogenins obtained from sisal waste

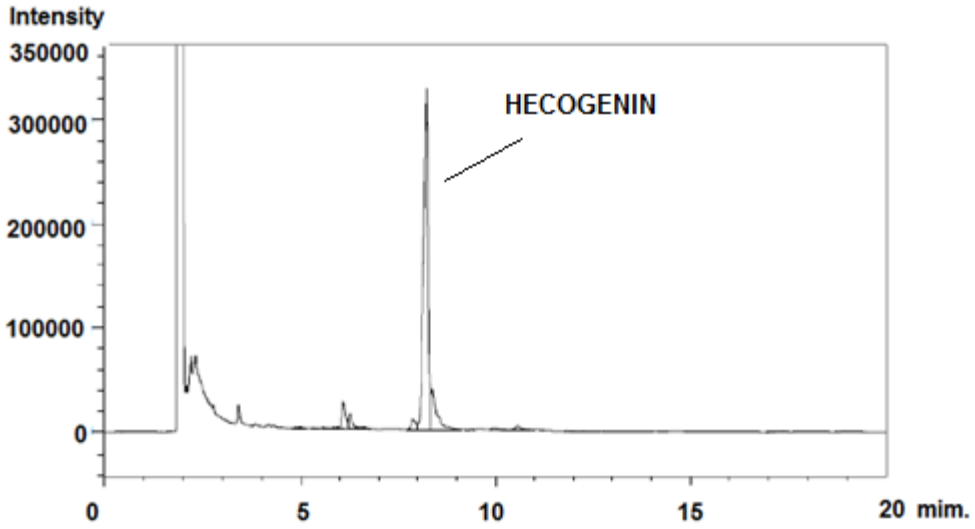

Fig. 5. GC chromatogram of acid-hydrolysed ethyl acetate sisal waste extract

CONCLUSIONS

1. Liquid-liquid extraction can be successfully employed in sisal waste for the extraction of pure hecogenin, which can be subsequently converted into derivatives that are important in the pharmaceutical industry. Notably, the recovery of hecogenin using liquid-liquid extraction was found to be a simple process.
2. We believe this method, based on simple liquid-liquid extraction, is applicable to investigate the plants and microorganisms that produce glycosylated steroid.

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